

Correspondences

Nitric oxide synthase is not essential for *Drosophila* development

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Nitric oxide (NO) is a key regulator of diverse biological processes, including the modulation of blood vessel tone [1]. Nitric oxide synthase (NOS), which oxidizes arginine to produce NO and citrulline [2], is found in organisms from bacteria to humans. Despite the impact of NO on physiology, mice lacking all three mammalian NOS isoforms develop to term and are viable [3]. There is a single NOS ortholog encoded in the *Drosophila* genome (*Nos*). Regulski *et al.* [4] described a mutation in a conserved residue that abrogates NOS activity, and reported that this lesion confers lethality (*Nos^C*). However, two lines of evidence led us to believe that this lethality could be due to a closely associated mutation rather than the lesion in *Nos* itself. First, the lethality was not rescued by reintroduction of NOS. Second, while the authors convincingly demonstrate that they have generated a mutation in the *Nos* gene that inactivates the enzyme, they do so for only one of the 17 alleles that they assign to the *Nos* complementation group. Beginning with a stock of *Nos^C* provided by Regulski *et al.* [4], we isolated recombinant chromosomes in which we separated the lethal lesion from the point mutation in *Nos^C*. Additionally, we generated a deletion that removes significant portions of the *Nos* coding sequences, including those responsible for synthesis of NO, and found it to be homozygous viable. Both our deletion and *Nos^C* eliminate NOS enzymatic activity without affecting *Drosophila* development, and without obviously compromising the health of the flies.

We created a transgenic line carrying the *Nos* cDNA (Dijkers and O'Farrell, unpublished data). Its

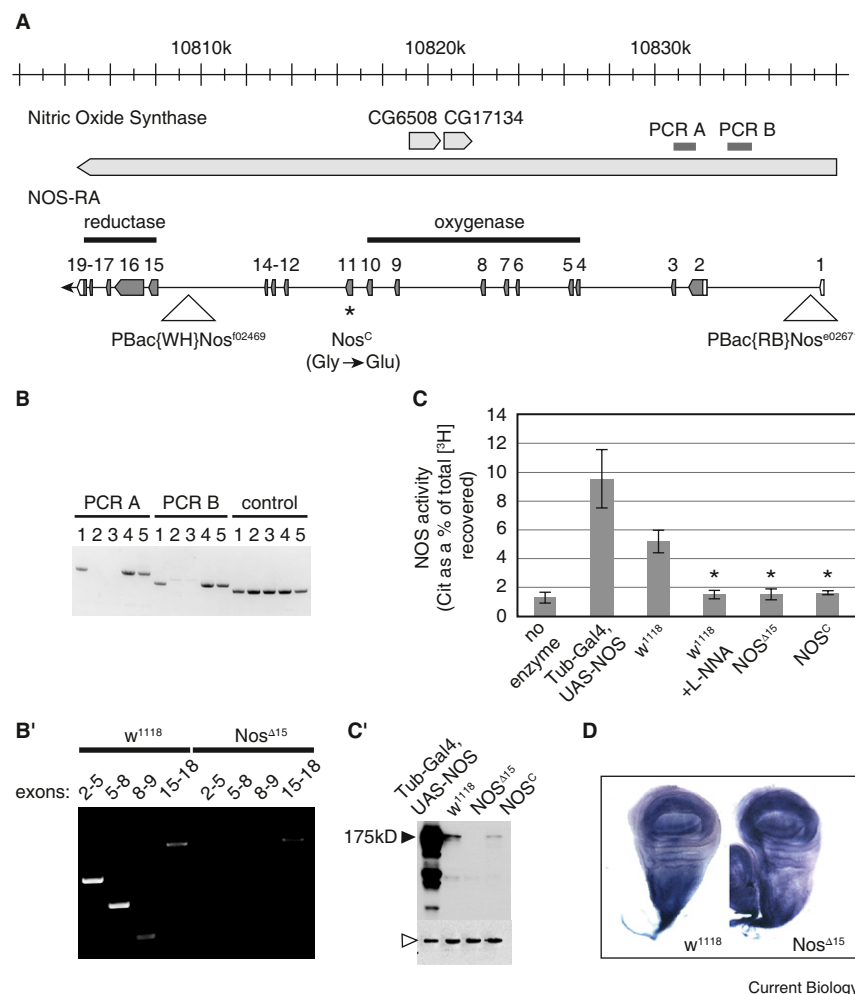


Figure 1. Characterization of a deletion of the *Nos* oxygenase domain.

(A) The *Drosophila* Nitric Oxide Synthase genomic region. Transcript NOS-RA with its exons enumerated and its coding region shaded (transcript orientation from right to left follows the genomic map). *Nos^{Δ15}* is a deletion of the region between *PBac{WH}Nos¹⁰²⁴⁶⁹* and *PBac{RB}Nos¹⁰²⁶⁷¹*. *Nos^{Δ15}* also deletes *CG6508* and *CG17134* (depicted). Since the deletion exhibits no evident phenotype, these two coding sequences also appear dispensable. The asterisk represents the point mutation associated with *Nos^C*. (B) PCR amplification of genomic sequences PCR A and B, and a control region that lies outside the *Nos* locus. Shown here are five candidate recombinants; candidates two and three are deleted for the region between *PBac{WH}Nos¹⁰²⁴⁶⁹* and *PBac{RB}Nos¹⁰²⁶⁷¹*. (B') RT-PCR directed against the indicated exons confirmed the absence of the oxygenase domain in *Nos^{Δ15}*; a transcript that includes exons 15–18 is expressed in the deletion strain. (C) NOS activity of extracts from adult heads; activity is represented by the amount of [³H]citrulline as a percent of the total [³H] recovered (arginine + citrulline); L-NNA is the NOS inhibitor, N^G-nitro-L-Arginine; asterisk indicates significant reduction in NOS activity compared to *w¹¹¹⁸* uninhibited control ($p < 0.005$). (C') Western blotting of the extracts used for NOS activity assays reveals the levels of NOS (black arrowhead); β-Tubulin is used as a loading control (white arrowhead). Over-expression of NOS driven from the transgene results in several minor bands that are smaller than the main band at 175 kD. These bands are not visible in other sample types and therefore are unlikely to correspond to endogenously produced isoforms. (D) NADPH-dia-phorase reactivity in *w¹¹¹⁸* and *Nos^{Δ15}* wing discs.

expression under the control of a ubiquitous promoter produced a NOS immuno-reactive band at 175 kD (Figure 1C'), but had no obvious effect on the flies. Expression of this transgene did not rescue the lethality associated with the original *Nos^C* chromosome. While it is possible that

the transgene is not expressed in a way that is spatially and temporally appropriate for complete rescue, its expression did not even modify the stage of lethality, suggesting that the lethality associated with *Nos^C* is not the direct consequence of a deficiency in NOS function.

Since *Nos^C* was selected as a lethal over a deletion that removed five nearby open reading frames (ORFs) as well as *Nos* 5' sequences, it seemed possible that a second change in one of these five ORFs might be the cause of the lethality associated with *Nos^C*. Such a closely linked secondary mutation would not have been easily separated by backcrossing. We exploited an insertion element within the *Nos* locus (*PBac{WH}Nos^{f02469}*, Figure 1A) to isolate viable recombinant chromosomes that were likely to retain the *Nos^C* mutation. Sequence analysis confirmed that the newly isolated chromosomes carry the substitution described by Regulski *et al.* [4]. We obtained three recombinant chromosomes, giving a separation of roughly 0.08 map units (~22 kb) between the lethal and the *Nos^C* lesion (Supplemental Data). Importantly, flies homozygous for this purified *Nos^C* isolate are viable.

To further test the requirement for NOS, we produced a new mutation by inducing directed recombination events between two insertion elements, one that is upstream of the *Nos* protein coding sequence (*PBac{RB}Nos^{e02671}*) and one that is between the fourteenth and fifteenth exons (*PBac{WH}Nos^{f02469}*) (Figure 1A). We isolated candidate recombinant chromosomes without selection, and used PCR to identify three lines that carry deletions within *Nos* (Figure 1A,B). One of these, which we refer to as *Nos^{Δ15}*, was selected for further analysis. *Nos^{Δ15}* homozygotes produced a truncated RNA corresponding to the 3' half of the coding sequence, as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1B). However, this transcript lacks translation start sequences and we detected no protein product (Figure 1C). We cannot rigorously eliminate the possibility that these residual sequences encode a vital function, but these data suggest it is unlikely. Notably, flies that are homozygous for this deletion are viable and display no discernible defects. Furthermore, flies carrying *Nos^{Δ15}* in transheterozygous combination with *Nos^C* are similarly viable.

The *Nos^{Δ15}* deletion removes sequences encoding residues 1–757, encompassing the entire oxygenase domain and including regions that

bind the catalytic heme and the substrate (arginine). These sequences produce a protein structure that is uniquely dedicated to NOS catalytic activity [5,6]. We wanted to confirm the functional disruption of NOS activity in both *Nos^{Δ15}* and in our new isolate of *Nos^C*. NOS generates NO through the stoichiometric conversion of L-arginine to L-citrulline [2]. We assayed extracts prepared from homozygous *Nos^{Δ15}* and *Nos^C* adult heads for their abilities to stimulate this conversion. NOS enzymatic activity was reduced in both *Nos^{Δ15}* and *Nos^C* homozygotes to a level comparable to negative controls that lacked extract or included a NOS inhibitor (Figure 1C).

To generate NO from L-arginine, NOS reduces and activates its catalytic heme by oxidizing NADPH. The diaphorase reaction reveals oxidation of NAPH histochemically. Although numerous oxidoreductases catalyze NADPH oxidation, diaphorase staining is often attributed specifically to NOS. This is based on the observation that diaphorase staining and NOS distribution are coincident in the mammalian brain, particularly in tissue that is fixed with paraformaldehyde [7,8]. Interestingly, we found that NADPH-diaphorase staining in *Nos^{Δ15}* wing discs (Figure 1D) and gut (not shown) is comparable to wild type, indicating oxido-reductase activity remains in these NOS-deficient tissues. This activity might represent any of a variety of oxido-reductases. Indeed, even though we did not detect the protein product, it is possible that low levels of expression of the carboxy-terminal reductase domain of NOS, which we did not delete, contribute to this activity. However, regardless of the source of this activity, it occurs in the absence of the NOS catalytic center and NO production. These results underscore the caveats associated with the use of the NADPH-diaphorase reactivity as a measure of NOS activity.

Our results show that the recognized *Drosophila* orthologue of NOS is non-essential. The absence of any other *Drosophila* sequence related to the NOS catalytic domain suggests that NOS activity itself is non-essential. Since NO can be produced by NOS-independent reduction of nitrite, a reaction carried out by microbes and

eukaryotic mitochondria, it remains possible that NO has an important role in *Drosophila* despite NOS dispensability [9]. NOS is conserved from prokaryotes [10] to humans. This preservation during evolution suggests a role for the enzymatic activity, but it is dispensable at least in the context of unperturbed *Drosophila* development in the lab environment.

Supplemental Data

Supplemental data are available at [http://www.cell.com/current-biology/supplemental/S0960-9822\(09\)02130-7](http://www.cell.com/current-biology/supplemental/S0960-9822(09)02130-7)

Acknowledgements

We would like to thank S. Deluca, J. Farrell, M. McClelland and J. Ward for critically reading the manuscript. E.A.S. is supported by a fellowship from the Canadian Institutes of Health Research. The work was supported by a grant from the National Institutes of Health GM08654 to P.H.O'F.

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